Fluorescence Measurements in Multi-FRET Systems

1 Model

The model is a simple rate balance equation on each excited fluorphore species. Thus we may write for excited fluorphore species X_n:

$$\frac{dX_n}{dt} = \epsilon_n \Gamma - k_n X_n + \sum_{m=donor} k_{mn} X_m - \sum_{o=acceptor} k_{no} X_n \quad , \tag{1}$$

where ϵ_n = the extinction coefficient at the excitation wavelength, Γ = constant factor depending on excitation light and the geometry used, k_n = radiative and all other non-FRET decay rates (inter system crossing, quenching, internal conversion) and k_{mn} = FRET transfer from m (donor) to n (acceptor). $\Gamma\epsilon_n$ gives the number of excited fluorphore species created per unit time by excitation light

The actual fluorescence from this fluorphore is proportional to $X_n k_n \phi_n$ where ϕ_n is the quantum yield of the fluorphore. Let us study the consequences of these equations for a system of two fluorphores:

2 Two Fluorphores

Consider two fluorphores X_1 (donor) and X_2 (acceptor). Assume a steady state so the time derivative drops out and we get

$$0 = \epsilon_1 \Gamma - k_1 X_1 - k_{12} X_1 \qquad 0 = \epsilon_2 \Gamma - k_2 X_2 + k_{12} X_1 \tag{2}$$

$$\begin{array}{ccc} X_1 & \xrightarrow{k_{12}} & X_2 \\ {\scriptstyle k_1}\downarrow & & \downarrow{\scriptstyle k_2} \end{array} \tag{3}$$

The efficiency for FRET may be written as

$$\eta_{12} = \frac{k_{12}}{k_{12} + k_1} \tag{4}$$

We can use this equation to eliminate the rates in expressions for fluorescence. Let the efficiency of collecting fluorescence light be given by f_1 and f_2 for the two fluorphores. So we may write fluorescence of fluorphore X_1 is given by:

$$k_1 X_1 \phi_1 f_1 = \Gamma \epsilon_1 (1 - \eta_{12}) \phi_1 f_1 \tag{5}$$

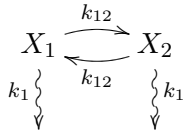
And the expression for the acceptor fluorphore is then:

$$k_2 X_2 \phi_2 f_2 = \Gamma(\epsilon_2 + \epsilon_1 \eta_{12}) \phi_2 f_2 \tag{6}$$

In these expressions we may take the fluorescence to mean fluorescence over the entire range the fluorphore emits. However in practice it is possible to use just the fluorescence intensity at a given wavelength. The entire spectrum is only needed for the curve fitting procedure described in the paper. Now we are in a position to compare these expressions to those used conventionally in fluorescence. The easiest way to measure efficiencies involves measuring fluorescence of donor in the presence of acceptor (F_a) and in the absence of acceptor (F_{na}) .The expression used is:

$$\eta_{12} = 1 - \frac{F_a}{F_{na}} \tag{7}$$

It is easy to see by visual inspection that this will be expression we get from the model too. Now let us take the case when the two fluorphores are the same. Here there will be two extra terms in the equations because now both fluorphores can be donors as well as acceptors. We assume that the constants associated with each fluorphore are the same.



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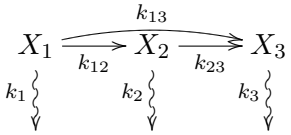
$$0 = \epsilon_1 \Gamma - k_1 X_1 - k_{12} X_1 + k_{12} X_2 \qquad 0 = \epsilon_1 \Gamma - k_1 X_2 + k_{12} X_1 - k_{12} X_2 \tag{9}$$

The symmetry in the equations means that $X_1 = X_2$. Both of them are equal to the value for a free fluorphore. Evidently the backward and forward transfers cancel each other out.

$$X_1 = X_2 = \frac{\Gamma \epsilon_1}{k_1} \tag{10}$$

3 Three Fluorphores



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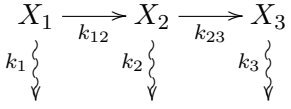
Consider a system of three fluorphores and follow the procedure outlined earlier. If the fluorphores are X_1,X_2,X_3 then we may write the following system of equations:

$$\begin{aligned}
\frac{dX_1}{dt} &= \epsilon_1\Gamma - k_1X_1 - k_{12}X_1 - k_{13}X_1 \\
\frac{dX_2}{dt} &= \epsilon_2\Gamma - k_2X_2 + k_{12}X_1 - k_{23}X_2 \\
\frac{dX_3}{dt} &= \epsilon_3\Gamma - k_3X_3 + k_{13}X_1 - k_{23}X_2
\end{aligned} \tag{12}$$

Now we assume a steady state so the give derivatives become equal to zero. We can rewrite these equation in the following instructive way:

$$\begin{aligned}
0 &= \epsilon_1\Gamma - (k_1X_1) - \frac{\eta_{12}}{1-\eta_{12}}(k_1X_1) - \frac{\eta_{13}}{1-\eta_{13}}(k_1X_1) \\
0 &= \epsilon_2\Gamma - (k_2X_2) + \frac{\eta_{12}}{1-\eta_{12}}(k_1X_1) - \frac{\eta_{23}}{1-\eta_{23}}(k_2X_2) \\
0 &= \epsilon_3\Gamma - (k_3X_3) + \frac{\eta_{13}}{1-\eta_{13}}(k_1X_1) + \frac{\eta_{23}}{1-\eta_{23}}(k_2X_2)
\end{aligned} \tag{13}$$

Note that for the first fluorphore we have assumed it has a FRET interaction with two fluorphores simultaneously. So we can no longer think of efficiency in the way described in equation 4. But it is still better to use the two fluorphore efficiencies because they are simpler to define and use. Solving these equation leads to a complicated solution which does not provide much information. Let us take the simpler case when we can neglect FRET transfer from the first fluorphore to the third fluorphore. This is the case of our experiments We can write the solution in terms of measurable quantities as shown below.



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$$\begin{aligned}
k_1X_1 &= \Gamma(\epsilon_1 - \eta_{12}\epsilon_1) \\
k_2X_2 &= \Gamma(\epsilon_2 - \eta_{23}\epsilon_2 + \eta_{12}\epsilon_1 - \eta_{12}\eta_{23}\epsilon_1) \\
k_3X_3 &= \Gamma(\epsilon_3 + \eta_{23}\epsilon_2 + \eta_{12}\eta_{23}\epsilon_1)
\end{aligned} \tag{15}$$

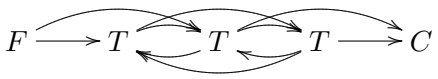
Multiplying each of these by the quantum yield of the fluorphore gives us the fluorescence.

4 Calculations

The equations listed above were used for calculations in the paper. In particular using the TAMRA fluorphore fluorescence from molecules FTC and FT_ we could easily measure the TAMRA to Cy5 fluorphore efficiency. Then we used absorption measurements to obtain numbers $\epsilon_T/\epsilon_F = 0.11$ and $\epsilon_C/\epsilon_F = 0.03$. To get the efficiency of FAM to TAMRA transfer we compared fluorescence from Cy5 fluorphore in FTC and _TC. The ratio of fluorescence can be obtained by the curve fitting procedure described in the paper and then normalizing to insure equal concentrations. We can write the ratio of fluorescence from the Cy5 fluorphore in the two molecules as:

$$\frac{C_{FTC}}{C_{\_TC}} = \frac{\epsilon_C^{bf} + \eta_{TC}^{bf}\epsilon_T^{bf} + \eta_{FT}^{bf}\eta_{TC}^{bf}\epsilon_F^{bf}}{\epsilon_C^{bf} + \eta_{TC}^{bf}\epsilon_T^{bf}} \tag{16}$$

Here the superscript bf denotes Bi-FRET molecules and the subscript gives the fluorphore involved. This equation allows us to calculate the FAM to TAMRA efficiency. Note that transfer from FAM to Cy5 is insignificant because of the large separation in their spectra. In the Quad-FRET case we used the same balance equation. However things are more complicated as shown in the diagram.



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Using the fluorescence from FTTTC, _TTTC and FT_TC we can obtain 8 different fluorescence measurements - a signal from every fluorphore present. However we need the quantum yields to cancel out and this reduces the numbers we can use to 5 - the ratios of the fluorescence from the same fluorphore in FTTTC, _TTTC or FT_TC. We have 6 variables to consider - the six possible efficiencies- FAM to TAMRA, TAMRA to Cy5, TAMRA to TAMRA over 10 and 20 base pairs. There is a relationship between the efficiency over 10 base pairs and 20 base pairs. In our calculations we assumed that in the TAMRA to TAMRA case the distance over 20 base pairs is twice that of over 10 base pairs - a reasonable assumption since the TAMRA fluorphores are attached to the same strand of the double stranded DNA and to the same base (thymine). We also assume that all the three TAMRA fluorphores behave similarly. To solve the equations we used a minimization procedure involving a global (that is between 0 and 1) brute force search for values of efficiencies that would minimize the relative square deviation from the measured relative fluorescence ratios. We located a global minimum at the values mentioned in the paper. We also found that changing the ratio of distance between FAM and TAMRA over 10 and 20 base pairs between 1.5 and 2 did not change the the efficiency values significantly. The same also applied for TAMRA to Cy5. This does however change the efficiencies that involve skipping a

fluorphore. The errors were calculated using error propagation From the efficiencies we can obtain the time constant for FRET transfer in terms of the fluorescence life time of the free donor dye. The energy transfer process on the TAMRA dyes is similar to that of a random walk with a reflecting barrier at one end and an absorbing barrier at the other end. This allows an estimate on the average time for transfer of a signal.